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RESEARCH ARTICLE

High ESAT-6 Expression in Granuloma Necrosis Type of Tuberculous Lymphadenitis

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Abstract

A granuloma is one of host cellular immune response form to intracellular and persistent pathogens, and result in the aggregation of several activated immune cells. Intracellular pathogens manipulate host immune responses to avoid immune reactions. *M. tuberculosis* is the intracellular and persister pathogen, which can stimulate granuloma formation. The formation this granulomas still have different opinions, whether it is the host's way to isolate *M. tuberculosis*, or how these pathogens are to escape immune responses. Early secretory antigenic target (ESAT)-6 is a typical secretory protein produced by the locus of the gene region of difference (RD)-1 *M. tuberculosis*. ESAT-6 plays a role in the immunopathogenesis of tuberculosis. This study aims to compare ESAT-6 antigen expression from *M. tuberculosis* between granulomas with necrosis and granulomas without necrosis. This study was an analytic observation study with a cross-sectional design. Forty-six lymph node paraffin blocks from tuberculous lymphadenitis patients in Department of Anatomical Pathology, Dr. Hasan Sadikin General Hospital, Bandung in 2017 were made in preparations and stained by hematoxylin eosin to assess the presence of necrosis in granulomas, immunohistochemical using ESAT-6 antibodies, then it was quantified using histoscore. Histoscore for ESAT-6 not normally distributed, so it uses Mann-Whitney test used. The results showed that there were 31 granulomas with necrosis (histoscore mean=27.6%) and 15 granulomas without necrosis (histoscore mean=15.1%), there was a significant difference with $p<0.05$ ($p=0.03$). The conclusion of this study there is a high histoscore ESAT-6 expression in granuloma type of necrosis tuberculous lymphadenitis.

Keywords: ESAT-6, granuloma, necrosis

Ekspresi ESAT-6 Tinggi pada Granuloma Limfadenitis Tuberkulosis Tipe Nekrosis

Abstrak

Granuloma merupakan salah satu bentuk respons imun seluler pejamu terhadap patogen intraseluler. Patogen intraseluler memanipulasi respons imun pejamu untuk menghindari reaksi imun. *M. tuberculosis* adalah patogen intraseluler dan persister yang dapat menstimulasi pembentukan granuloma. Terbentuknya granuloma masih memberikan pendapat yang berbeda, apakah merupakan cara tubuh untuk mengisolasi *M. tuberculosis* atau cara patogen ini untuk menghindari respons imun. *Early secretory antigenic target* (ESAT)-6 adalah protein sekretori khas yang dihasilkan oleh lokus gen *region of difference* (RD)-1 *M. tuberculosis*. ESAT-6 berperan dalam imunopatogenesis tuberkulosis. Penelitian ini bertujuan menganalisis perbedaan ekspresi antigen ESAT-6 *M. tuberculosis* antara granuloma dengan nekrosis dan granuloma tanpa nekrosis. Penelitian ini merupakan penelitian observasi analitik dengan desain *cross sectional*. Blok parafin kelenjar getah bening didapat dari pasien yang didiagnosis limfadenitis tuberkulosis di Departemen Patologi Anatomi, RSUP Dr. Hasan Sadikin Bandung pada tahun 2017. Blok parafin tersebut dibuat blangko preparat dan diwarnai dengan hematoksilin eosin untuk menilai nekrosis pada granuloma serta imunohistokimia menggunakan antibodi ESAT-6. Kemudian, sediaan preparat imunohistokimia tersebut dikuantifikasi menggunakan metode *histoscore* sehingga didapatkan data berupa nilai skor dari pewarnaan ESAT-6. Selanjutnya, dilakukan uji beda antara *histoscore* granuloma dengan nekrosis dan granuloma tanpa nekrosis tersebut dianalisis karena nilai skor ESAT-6 berdistribusi tidak normal sehingga menggunakan uji Mann-Whitney. Hasil penelitian menunjukkan terdapat 31 granuloma dengan nekrosis (*histoscore* rerata=27,6%) dan 15 granuloma tanpa nekrosis (*histoscore* rerata=15,1%), serta terdapat perbedaan signifikan dengan $p<0,05$ ($p=0,03$). Simpulan, ekspresi ESAT-6 tinggi pada granuloma limfadenitis tuberkulosis dengan nekrosis.

Kata kunci: ESAT-6, granuloma, nekrosis

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Introduction

Tuberculosis is still a high burden of disease in the world and in Indonesia.[1] Several factors influence this disease to have the impact on the treatment and prevention program.[2,3] Tuberculosis has several types with extrapulmonary tuberculosis incidence is about 20% of all tuberculosis. Lymphadenitis is the most extrapulmonary tuberculosis.[1]

A granuloma is one of host cellular immune response form to intracellular and persistent pathogens, and result in the aggregation of several activated immune cells. There are differences on the host's cellular immune response to different mycobacterium species, depending on their respective virulence. *Mycobacterium bovis* can modulate miR-155 expression in macrophages, through toll-like receptor 2 (TLR2) and NF-kB signals. The transduction signal will activate the apoptosis event of macrophage cells, as one of the pathways for cell death to eliminate intracellular *M. tuberculosis*. *M. tuberculosis* also induces the expression of miR-155 but differs in the final results of mycobacterium elimination. *M. tuberculosis* tends to be more resistant to macrophage apoptosis for the elimination of intracellular mycobacterium, compared to *M. bovis*. The host immune response to *M. smegmatis* is the same as that of *M. bovis*. *M. bovis* and *M. smegmatis* are less virulent compared to *M. tuberculosis*. This phenomenon shows that *M. tuberculosis* causes failure of the innate immune response to eliminate *M. tuberculosis*, so it is more likely to progress to adaptive immune response.[3,4]

Adaptive immune response to *M. tuberculosis* different from another mycobacterium, through induction of T helper two cells (Th2). The infection becomes more progressive and can cause reactivation. Reactivation occurs through the destruction of a stable granuloma so that localized *M. tuberculosis* can come out of the granuloma confinement and become outside the cell. The extracellular *M. tuberculosis* induces the host's immune response. The results of *M. tuberculosis* reaction with the host's immune response cause the clinical condition of the infectious disease. *M. tuberculosis*, which is already outside the cell and granuloma, infects the tissue more widely and can spread through lymphogens and hematogenous.[5]

The cell population of granulomas consisting of giant Langerhans cells (GLCs) and epithelioid cell (EC) shows the host's immune response ability to infectious agents, in other words, the infectious agent is not too aggressive. Conversely, the picture of necrosis shows the aggressiveness of infectious agents.[6] Necrosis is one of the pathways of macrophage cell death, and shows the failure of macrophages to eliminate *M. tuberculosis*.[7–9] Necrosis is also a mechanism for cellular or slow-type hypersensitivity response, to eliminate intracellular bacteria.[10,11]

M. tuberculosis has an early secretory antigenic target-6 (ESAT-6) antigens. *M. bovis* BCG and *M. avium* do not own the antigen. Early secretory antigenic target-6 is secreted by *M. tuberculosis* so that it can be detected in the blood or granuloma tissue of active pulmonary tuberculosis patients.[1–3] ESAT-6 can be detected in tuberculous granuloma lymphadenitis, and immunohistochemical staining for ESAT-6 antigen has been shown to provide 88.6% sensitivity.[4]

ESAT-6 plays a role in the immunopathogenesis of tuberculosis.[2,5] Several studies have proven that ESAT-6 plays a role in the immune response to mycobacterium, including its influence on the expression of IL-6, IL-8, TNF-α by macrophages.[3,6–8] Studies in vitro on macrophage cell cultures proven that ESAT-6 can modulate the expression of microRNA-155 through receptors TLR1 or TLR6.[5] MicroRNA-155 through IL-6 can increase MMP-1, which contributes to granuloma rupture and causes necrosis. Based on the framework, this study aims to analyze the role of ESAT-6 in granulomas in tuberculous lymphadenitis.

Methods

The material used in this study is stored lymph nodes paraffin blocks from tuberculous lymphadenitis patients diagnosed histopathologically in Anatomical Pathology Department, Dr. Hasan Sadikin General Hospital Bandung in 2017. The inclusion criteria were as follows; paraffin block stored less than four years, and tissue volume was sufficient for research and archives. The exclusion criteria are paraffin blocks derived from patients diagnosed with diabetes mellitus, positive HIV, and immunodeficiency.

Paraffin blocks that are suitable for the inclusion and exclusion criteria were sliced using microtomes and preparations were made and stained using ESAT-6 immunohistochemistry and hematoxylin eosin. The preparation observed under a light microscope and ESAT-6

quantification used histoscore. The histoscore value was the result of the multiplication between the intensity of the brown color and the color distribution. Brown intensity consists of 3 scores: 1 for low intensity, 2 for moderate intensity and 3 for high intensity. The brown color distribution scores consists of; score 1 (0–20%), score 2 (>20–50%), score 3 (>50–80%), and 4 (>80–100%).

Type of granuloma, the presence of granuloma and necrosis area observed from the preparations stained with hematoxylin eosin. Determination of histoscore and histopathology conducted by three observers, consisting of the anatomical pathologist and two researchers. Statistical analysis was done using Mann-Whitney test.

This study has approved by Health Research Ethics Committee, Faculty of Medicine, Universitas Padjadjaran, Bandung number: 1050/UN6.C10/PN/2017.

Results

Assessment of granuloma necrosis stained by hematoxylin eosin, in Figure 1A shows granulomas without necrosis, while Figure 1B shows granulomas with necrosis.

ESAT-6 appears as shown in Figure 2. The average ESAT-6 histoscore values for each group

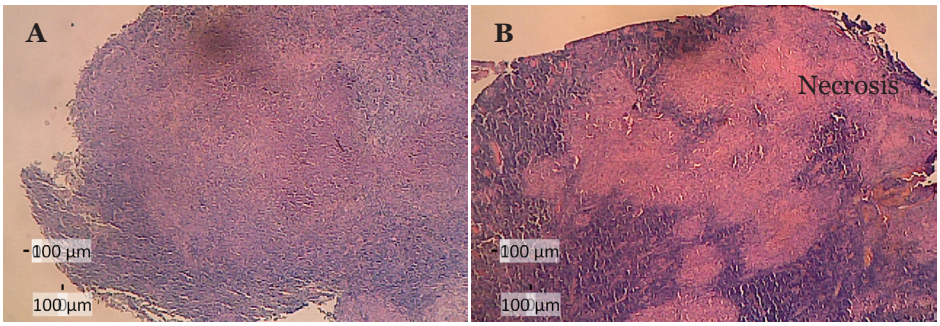

Figure 1 Hematoxylin Eosin Staining
A. Granuloma without necrosis (40×); B. Granuloma with necrosis (40×)

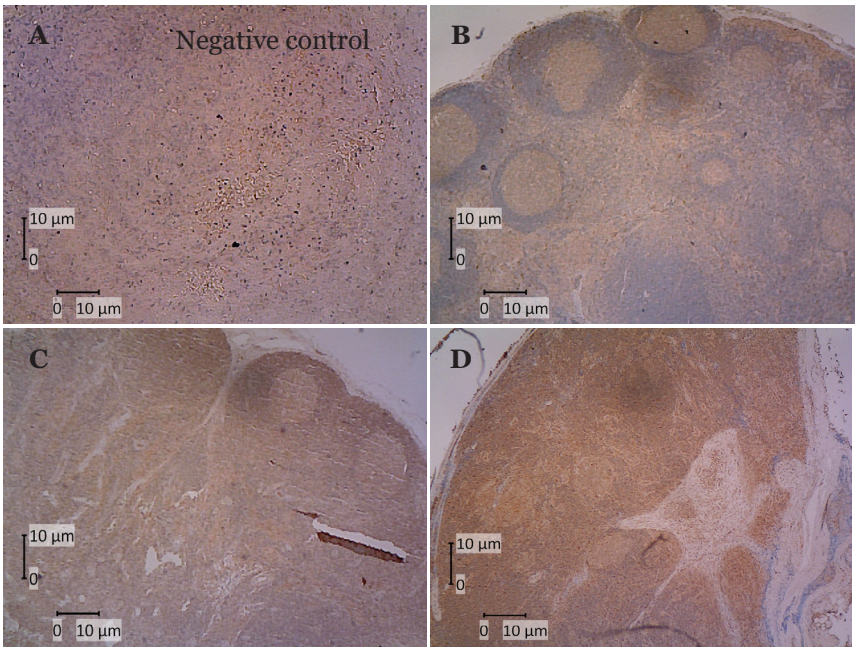

Figure 2 Early Secretory Antigenic Target (ESAT)-6 Staining
A. (100×) as a negative control, in granulomatous lymphadenitis shows negative ESAT-6 given a score of 0, low intensity; B. (100×) image scored 1; C. (100×) medium intensity image is given a score of 2, high intensity; and D. (100×) images, given a score of 3

Table Average ESAT-6 Histoscre

Necrosis	Average ESAt-6 Score	Sig*
Negative	15.1 (15)	0.03
Positive	27.6 (31)	
Total	100 (46)	

*Mann Whitney test (p<0.05, significant different)

seen in Table.

Discussion

A granuloma is an aggregation of some inflammatory cells, especially mature macrophages which form aggregates in response to an antigen. The antigens come from a bacterium, fungus, foreign body, and immune complex.[9] The purpose of forming a granuloma is to isolate the antigen from the host and facilitate eradication of the antigen. This essential immune reaction provides the body with protection from antigen recognition, very important in cases of mycobacterial infection. Immune disorders cause granuloma to form accompanied by necrosis. Granuloma occurs in TNF-α deficiency, interleukin-12 (IL-12), or interferon-gamma (IFN-γ).[10]

Assessment of cell populations against granulomas consisting of GLCs and EC shows the host's immune response ability to infectious agents, in other words, infectious agents are not too aggressive. On the other hand, the picture of necrosis shows the aggressiveness of the infectious agent.[1,12] Necrosis is one of the pathways of macrophage cell death and shows the failure of macrophages to eliminate *M. tuberculosis*.[5,7] Necrosis is also one of the mechanisms of slow cellular type or hypersensitivity response, to eliminate intracellular bacteria.[10,11] In this study granuloma assessment based on the presence of necrosis, without assessing other cellular components.

Histoscore ESAT-6 was higher in granulomas with features of necrosis, compared with granulomas without necrosis, caused by the high expression of ESAT-6 by *M. tuberculosis* or a large number of *M. tuberculosis*. Early secretory antigenic target-6 is a typical secretory protein produced by the locus of the gene region of difference-1 (RD-1) *M. tuberculosis* virulent isolate H37Rv.[12]

Early secretory antigenic target-6 usually found as a complex with culture filtrate protein (CFP)-10, has a molecular weight of 27 kDa and is composed of two mycobacterial proteins namely ESAT-6 (Rv3875 or EsxA) with a molecular weight of 6 kDa and CFP-10 (Rv3874 or 2-4EsxB) with a molecular weight of 10 kDa.[12]

M. tuberculosis has 22 pairs of genes from 11 genomic loci in the region of difference (RD)-1 4.5 which codes the ESAT-6/CFP-10 antigen family.[7] ESAT-6 secretion is carried out actively, using energy from adenosine triphosphate (ATP) with the help of gene groups such as the ATPase Rv3977 and Rv3871 membrane complexes and the Rv3877 transmembrane protein. The functional structure of this complex in the secretion process is the CarmCFP-10 which will bind to the ATPase membrane complex and will be carried through the cytoplasmic membrane by transmembrane proteins so that the ESAT-6/CFP-10 complex will be secreted by *M. tuberculosis*.[13]

Early secretory antigenic target-6 in granuloma lymphadenitis is one evidence of *M. tuberculosis* and plays a role in the formation of necrosis in granulomas.

Conclusion

High histoscore ESAT-6 expression in granuloma type necrosis tuberculous lymphadenitis, the presence of necrosis in granulomas indicates a diagnosis for lymphadenitis caused by *M. tuberculosis*.

Conflict of Interest

There was not a conflict of interest in this article.

Acknowledgement

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